VIROLOGY 222, 169–175 (1996)
ARTICLE NO. 0407

In Vitro Transcripts from Cloned cDNAs of the Lettuce Infectious Yellows Closterovirus

VICKI A. KLAASSEN,[1] DENNIS MAYHEW,* DEBORAH FISHER,* and BRYCE W. FALK

*Department of Plant Pathology, University of California, Davis, California 95616; and *Plant Pest Diagnostics Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, California 95832*

Received April 17, 1996; accepted May 28, 1996

Full-length cloned cDNAs of lettuce infectious yellows closterovirus (LIYV) RNAs 1 and 2 were constructed and fused to the bacteriophage T3 RNA polymerase promoter. To assess RNA replication, *Nicotiana benthamiana* protoplasts were inoculated with LIYV virion RNAs and LIYV cDNA-derived *in vitro* transcripts. Analysis of protoplasts inoculated with LIYV virion RNAs or capped (m^7GpppG) *in vitro* transcripts from LIYV RNA 1 and 2 cDNAs showed accumulation of LIYV genomic and putative subgenomic RNAs (sgRNAs), synthesis of LIYV coat protein, and formation of LIYV virions. Furthermore, protoplasts inoculated with only capped *in vitro* transcripts from LIYV RNA 1 cDNA showed accumulation of LIYV RNA 1 and its putative sgRNA, indicating that LIYV RNA 1 can replicate in the absence of LIYV RNA 2. Conversely, accumulation of LIYV RNA 2 was not detectable in protoplasts inoculated with only LIYV RNA 2 cDNA-derived capped *in vitro* transcripts. These data demonstrate that LIYV genomic RNAs are competent for replication in mesophyll protoplasts and that infectious *in vitro* transcripts can be derived from the cloned cDNAs of a closterovirus genome.

INTRODUCTION

Closteroviruses are widespread and economically important pathogens affecting a variety of plants, including many woody perennial (e.g., citrus and grapes) and herbaceous (vegetables and sugarbeets) crop plants (for reviews, see Coffin and Coutts, 1993, and Dolja *et al.,* 1994). In addition, closteroviruses display several distinct biological traits when compared to most other plant viruses. First, closteroviruses are distinguished from other rod-shaped plant viruses by their long (700–2000 nm), highly flexuous and filamentous virions (Tollin and Wilson, 1988). Second, closteroviruses are generally not mechanically transmissable, but are transmitted from plant-to-plant only by specific phloem-feeding insect vectors in a semipersistent manner (Falk and Duffus, 1988; Murant *et al.,* 1988). As a group, closteroviruses are transmitted by a variety of insect vectors, including aphids, whiteflies, and mealybugs. Third, unlike most plant viruses, closterovirus replication and movement is generally confined to plant host phloem cells (Murant *et al.,* 1988; Candresse and Martelli, 1995). Within phloem cells, closterovirus virions accumulate to form diagnostic particle aggregates (Lesemann, 1988). In addition, specific intracellular inclusions such as small cytoplasmic vesicles are often characteristic of closterovirus infection (Lesemann, 1988).

Closterovirus-specific characteristics, including their long filamentous virions, phloem tropism, and obligatory insect transmission have hindered the study of closterovirus molecular biology. Only recently have the complete nucleotide sequences of three closterovirus genomes [beet yellows closterovirus, (BYV; Agranovsky *et al.,* 1994), lettuce infectious yellows closterovirus (LIYV; Klaassen *et al.,* 1995), and citrus tristeza closterovirus (CTV; Karasev *et al.,* 1995)] been determined. These data further support the relative complexity of closteroviruses, demonstrating that their genomes are unique in both the quantity and quality of genetic material they contain. The LIYV, BYV, and CTV genomes are the largest (15,311, 15,480, and 19,296 nucleotides, respectively) of all positive-sense single-stranded RNA (ssRNA) plant viruses sequenced to date. Among positive-sense ssRNA viruses in general, only animal viruses of the *Coronaviridae* have genomes similar in size to these three closteroviruses (Candresse and Martelli, 1995). Computer-assisted analyses of the BYV, LIYV and CTV genomes have identified two conserved gene modules. The first or core module is conserved throughout viruses in the alphavirus supergroup (Koonin and Dolja, 1993) and encodes putative products whose deduced amino acid sequences include the principal replicative domains [methyltransferase (MET), helicase (HEL), and RNA-dependent RNA polymerase (RDRP); Agranovsky *et al.,* 1991a, 1994; Klaassen *et al.,* 1995; Karasev *et al.,* 1995)]. The second gene module is unique to the closteroviruses and includes genes encoding a small hydrophobic protein, a HSP70 homologue, a protein with a M_r of approximately 60,000, a diverged copy of the coat protein, and the coat protein

[1] To whom correspondence and reprint requests should be addressed.

0042-6822/96 $18.00

(Dolja *et al.,* 1994). While this second module is so far closterovirus-specific, the presence of genes encoding a HSP70 homologue and a diverged copy of the coat protein are most notable. Closteroviruses are the only viruses (of plants or animals) known to encode a HSP70 homologue (Agranovsky *et al.,*1991a,1991b), even though HSP70 proteins (commonly referred to as molecular chaperones) are ubiquitous proteins and are found in all types of cells from prokaryotes to eukaryotes (for a review, see Rothman, 1988). Karasev *et al.* (1992) demonstrated that the BYV-encoded HSP70 homologue binds microtubules *in vitro,* a property shared with some cellular HSP70s (Green and Liem, 1989). This supports the suggestion that the viral-encoded HSP70 may mediate the transport of virus-specific ribonucleoproteins (Agranovsky *et al.,* 1991b). In addition to the unique viral-encoded HSP70 homologue, closteroviruses are the only RNA plant viruses with elongated particles known to encode a diverged copy of the coat protein (Boyko *et al.,* 1992). Agranovsky *et al.* (1995) recently demonstrated that the BYV-diverged coat protein is located in a 75-nm segment at one end of the 1370-nm filamentous virion. Thus, closterovirus virions, unlike other filamentous virions of plant viruses, have a morphologically polar structure composed of two coat proteins.

The functional significance of large closterovirus genomes or the biological roles of the HSP70 homologue and the diverged copy of the coat protein cannot be determined until a suitable genetic system is developed. The availability of infectious *in vitro* transcripts from the cloned cDNA of viral RNAs has facilitated defining gene function by using a mutagenesis and reverse genetics approach (for a review on infectious transcripts of RNA viruses, see Boyer and Haenni, 1994). As a genetic system, the LIYV genome has several advantages over the BYV and CTV genomes. First, the LIYV genome is bipartite, consisting of LIYV RNAs 1 (8118 nucleotides) and 2 (7193 nucleotides; Klaassen *et al.,* 1995). The relatively smaller size of the two LIYV RNAs, compared to the large monopartite RNA genomes of BYV and CTV, will facilitate cloning full-length cDNA and synthesizing *in vitro* transcripts. Second, the putative principal replicative proteins are encoded in LIYV RNA 1 while the gene module unique to closteroviruses is included in LIYV RNA 2 (Klaassen *et al.,* 1995). The separation of these two gene modules will facilitate further analyses aimed at determining what products are necessary for LIYV RNA 1 and 2 replication.

In this paper, we report the construction of clones which contain full-length cDNA copies of LIYV RNAs 1 and 2. We have determined that LIYV virion RNAs and LIYV cDNA-derived *in vitro* transcripts replicate efficiently and are encapsidated in *Nicotiana benthamiana* protoplasts. In addition, we have used the infectious transcripts to assess the independent replication of LIYV RNAs 1 and 2 in protoplasts.

MATERIALS AND METHODS

Construction of full-length LIYV RNA 1 and 2 cDNA clones

Reverse transcription and polymerase chain reactions (PCR; Saiki *et al.,* 1988) were used to synthesize and amplify cDNA representing the 5′-terminal region of LIYV RNA 1 and full-length LIYV RNA 2 as shown in Fig. 1. Purified LIYV virion RNAs (ca. 100 ng; Klaassen *et al.,* 1994) and oligonucleotide Rmm502 (5′-TCAGCAAAGACGTCGAGCCA-3′, complementary to LIYV RNA 1 nucleotides 1130–1149) were heated at 80° for 5 min in moloney murine leukemia virus reverse transcriptase (M-MLV RT; BRL) buffer and then incubated at 57° for 45 min. M-MLV RT was used according to the manufacturer's protocol to synthesize LIYV RNA 1 cDNA corresponding to nucleotides 1–1149. One-fifth of the first strand cDNA reaction was used in combination with a second oligonucleotide, Rmm501T3 [5′-AACTGCAG*AATTAACCCTCACTAAAGG*TAAAGTTATACATTTACC-3′, including a *Pst*I site (bold nucleotides), the bacteriophage T3 RNA polymerase promoter (T3 promoter) sequence (nucleotides in italics), and the twenty 5′-terminal nucleotides of LIYV RNA 1 (nucleotides underlined)], Rmm502, and PCR to synthesize and amplify double-stranded cDNA representing the 5′-terminal 1149 nucleotides of LIYV RNA 1 (PCR 5′, Fig. 1A). The amplified product was purified using Qiaquick Spin Columns (Quiagen), digested with *Pst*I and *Hin*dIII and subcloned into the *Pst*I–*Hin*dIII sites of pSP55 (Klaassen *et al.,* 1995) to obtain a full-length copy of LIYV RNA 1 cDNA fused to the T3 promoter sequence. All plasmids were transformed into *Escherichia coli* DH5α. One clone, pSP9/55 (Fig. 1A), was selected for *in vitro* transcription reactions and subsequent analysis in protoplasts.

To synthesize full-length LIYV RNA 2 cDNA, purified LIYV virion RNAs (ca. 100 ng) were denatured with 10 m*M* methylmercuric hydroxide for 10 min at room temperature. First strand cDNA was synthesized using oligonucleotide Rmm501 [5′-ATAAGAATGCGGCCGCGGTCTAGTATACGAGATACA-3′, including a *Not*I site (nucleotides in bold) and complementary to the 20 3′-terminal nucleotides of LIYV RNA 2 (nucleotides underlined)], and Superscript II RNase H$^-$ Reverse Transcriptase (BRL) according to the manufacturer's recommendations. One-half of the first-strand cDNA reaction was used in combination with a second oligonucleotide, Rmm502T3 [5′-TGCAGAGCTC*AATTAACCCTCACTAAAGG*TAATCACAATTACCATTG-3′, including a *Sst*I site (nucleotides in bold), T3 promoter sequence (nucleotides in italics), and the twenty 5′-terminal nucleotides of LIYV RNA 2 (nucleotides underlined)], Rmm501, and the Expand Long Template PCR System (Boehringer Mannheim) to synthesize and amplify double-stranded cDNA of LIYV RNA 2 according to the manufacturer's recommendations. The amplified product was purified using Qiaquick Spin Columns

(Quiagen), digested with *Sst*I and *Not*I and ligated into *Sst*I–*Not*I-digested pSPORT-1 (BRL) to obtain a full-length copy of LIYV RNA 2 cDNA fused to the T3 promoter sequence. All plasmids were transformed into *E. coli* DH5α. One clone, pSP6 (Fig. 1B), was selected for *in vitro* transcription reactions and subsequent analysis in protoplasts.

The above cloning strategies were designed to place the T3 promoter sequence immediately upstream of LIYV RNA 1 and 2 cDNAs. The addition of nonviral sequences at the 5′-termini of LIYV RNA 1 and 2 cDNAs was unnecessary because the two 5′-terminal nucleotides of LIYV RNAs 1 and 2 (GG; Klaassen *et al.,* 1955) correspond with the +1 and +2 initiation sites required for efficient transcription by T3 RNA polymerase (Fig. 1C; Bailey *et al.,* 1983). *In vitro* transcripts are predicted to have 5′-termini identical to LIYV RNA 1 or 2, and two nonviral nucleotides at the 3′-termini (Fig. 1C).

In vitro transcription

Capped transcripts were synthesized from *Not*I-linearized pSP9/55 and pSP6 using the T3 mMessage mMachine kit (Ambion). The final cap analog [m^7(5′)-Gppp(5′)G]:GTP ratio was adjusted to 2.0:1. Uncapped transcripts were synthesized using *Not*I-linearized pSP9/55 or pSP6 and the T3 MEGAscript kit (Ambion). Following transcription, RNAs were analyzed on 0.8% nondenaturing agarose gels in 0.5× TAE (40 m*M* Tris–HCl, pH 7.9, 2.5 m*M* sodium acetate, 0.5 m*M* EDTA). Transcripts were stored at −70° or treated with DNase to remove the template DNA, extracted with phenol/chloroform, and recovered by ethanol precipitation.

Preparation and infection of *N. benthamiana* protoplasts

The methods used for *N. benthamiana* protoplast isolation and inoculation were as described by Jones *et al.* (1990) and Scholthof *et al.* (1993). Inoculations were performed using 5 μg purified LIYV virion RNAs or 10 μg *in vitro* transcripts of linearized pSP9/55 and/or pSP6 per 4.0×10^5 protoplasts.

Analysis of inoculated protoplasts

Protoplasts were pelleted by centrifugation (1310 *g*) and RNA was isolated using TRI Reagent (MRC) according to the manufacturer's recommendations. Total RNAs were denatured with glyoxal, separated by agarose gel electrophoresis, and transferred to Hybond N (Amersham) as previously described (Klaassen *et al.,* 1994). Immobilized LIYV RNA was detected by hybridization with DIG-labeled RNA probes (Boehringer Mannheim) according to the manufacturer's recommendations. Negative-sense DIG-labeled RNA probes were generated using SP6 RNA polymerase and linearized cDNA clones. The resulting probes were complementary to the 3′-terminal 938 and 1082 nt of LIYV RNAs 1 and 2, respectively. Positive-sense DIG-labeled RNA probes were generated using T3 RNA polymerase and linearized cDNA clones. The resulting probes were identical to the 5′-terminal 1064 and 1699 nt of LIYV RNAs 1 and 2, respectively.

To detect newly synthesized LIYV coat protein, protoplasts were pelleted by centrifugation (1310 *g*) 48 hr post-inoculation and disrupted by boiling in 1× Laemmli loading buffer (Laemmli, 1970) for 5 min. Proteins were separated by SDS–PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes for immunoblot analysis (Burnett, 1981). Blots were probed with antiserum to LIYV purified virions (Klaassen *et al.,* 1994) and serological reactions were detected using ECL Immunodetection (Amersham) according to the manufacturer's recommendations.

LIYV virus particles were detected in crude protoplast extracts by transmission electron microscopy. Protoplasts were pelleted by centrifugation (1310 *g*), resuspended in TE (10 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA) and ground with a plastic-tipped pestle. The grindate was diluted 1:50 in 2% phosphotungstic acid and applied to formar-coated electron microscope grids. Grids were viewed with a Zeiss CEM 902 electron microscope.

RESULTS

cDNA cloning and *in vitro* transcription of full-length LIYV RNAs 1 and 2

LIYV RNA 1 full-length cDNA clones were constructed by adding the 5′-terminal nucleotides to pSP55 as shown in Fig. 1A. However, we could not easily construct a full-length LIYV RNA 2 cDNA clone from existing clones, even though they represented most of LIYV RNA 2 (Klaassen *et al.,* 1995). Therefore, new full-length cDNA was synthesized using primers Rmm501 and 502T3, and RT-PCR (Fig. 1B). The synthesis of full-length LIYV RNA 2 cDNA was facilitated by denaturing LIYV virion RNAs with 10 m*M* methylmercuric hydroxide before first strand cDNA synthesis; the majority of resulting cDNA was full-length as determined by denaturing agarose gel electrophoresis (data not shown). In addition, cloning full-length LIYV RNA 2 cDNA was facilitated by amplifying cDNAs using PCR. Analysis of PCR products by gel electrophoresis demonstrated that the majority of amplified DNA was ca. 7200 bp, the expected size of full-length LIYV RNA 2 cDNA (data not shown). Approximately 10% of the resulting recombinant colonies contained plasmids with full-length LIYV RNA 2 cDNA.

Bioassays for LIYV RNAs and *in vitro* transcripts

Because LIYV is not mechanically transmissable to plants, we used a protoplast system for LIYV replication studies. Initial analyses were done using protoplasts made from a *Nicotiana tabacum* suspension cell culture

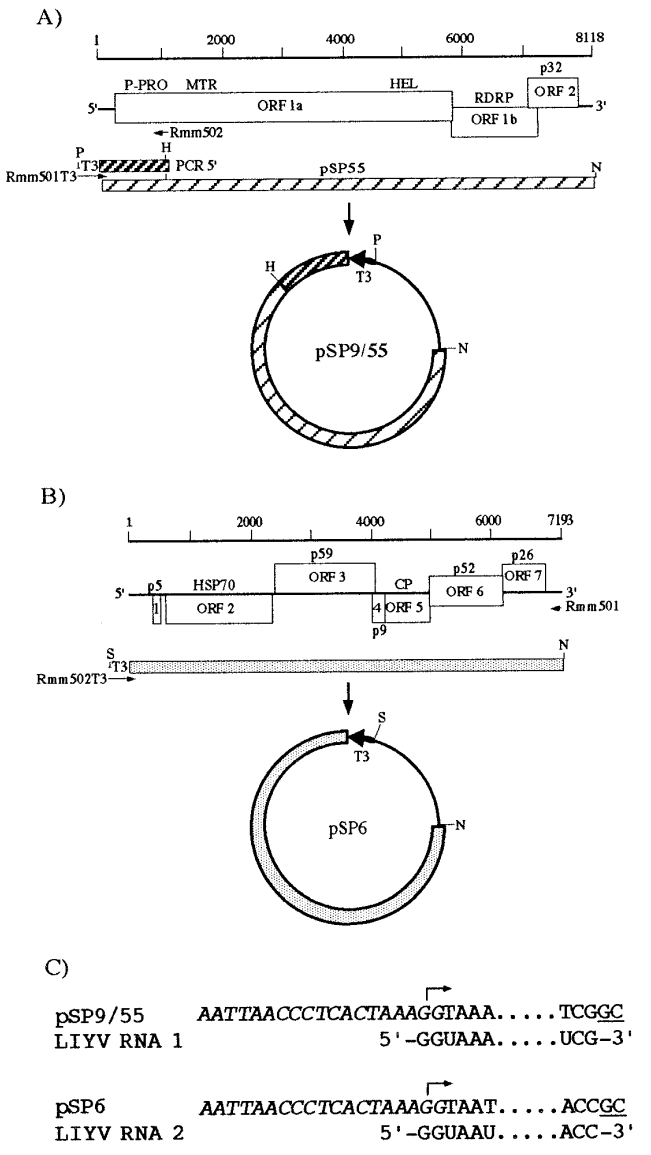

FIG. 1. Genome organization and construction of full-length cDNAs of lettuce infectious yellows closterovirus (LIYV) RNAs 1 (A) and 2 (B). Numbered lines represent nucleotide coordinates, beginning from the 5′-terminus for each RNA. Open rectangles depict open reading frames (ORFs) which are identified by conserved domains (P-PRO, papain-like protease; MTR, methyltransferase; HEL, RNA helicase; RDRP, RNA-dependent RNA polymerase; HSP70, HSP70 homologue; CP, LIYV coat protein; p52, duplicated diverged coat protein) or approximate molecular weights of putative products (p32, p5, p59, p9, and p26). Hatched rectangles below the LIYV RNAs represent PCR products and cDNA clones. The oligonucleotides used in cDNA synthesis and PCR are represented by horizontal arrows. The RNA promoter sequence used for *in vitro* transcription reactions and the restriction endonuclease sites used in cloning are indicated: T3, bacteriophage T3 RNA polymerase promoter sequence; P, *Pst*I; H, *Hin*dIII; S, *Sst*I; and N, *Not*I. (C) Comparison of the 5′-termini of LIYV genomic RNAs 1 and 2 with the bacteriophage T3 RNA polymerase promoter sequence (italicized) of cDNA clones pSP9/55 and pSP6, and the 3′-termini of LIYV genomic RNAs 1 and 2 with the 3′-termini of corresponding *in vitro* transcripts. The arrow depicts the transcription start site on the cDNA clones. *GC* indicates the two nonviral nucleotides (from a *Not*I site) at the 3′-termini of LIYV RNA 1 and 2 *in vitro* transcripts.

(Passmore *et al.,* 1993; Sanger *et al.,* 1994) and inoculated with LIYV virions and virion RNA. However, we were only able to detect the accumulation of positive- and negative-sense LIYV RNA 1 (Klaassen *et al.,* 1995). If LIYV RNA 2 was accumulating, it was to levels below our detection limit. Since *N. tabacum* is not reported to be a host for LIYV (Duffus *et al.,* 1986), we further assessed the replication of LIYV RNAs 1 and 2 in mesophyll protoplasts prepared from *N. benthamiana* plants, a systemic host for LIYV. Northern blot analyses of extracts from *N. benthamiana* protoplasts inoculated with LIYV virion RNAs showed the accumulation of both negative- and positive-sense LIYV RNAs 1 and 2, and putative subgenomic RNAs (sgRNAs; Fig. 2A, lanes 2 and 4). LIYV RNAs 1 and 2 were never detected in mock-inoculated protoplasts (data not shown).

Since the above results indicated that both LIYV RNAs 1 and 2 replicated efficiently in *N. benthamiana* protoplasts, we used these protoplasts to assay LIYV RNA 1 and 2 cDNA-derived *in vitro* transcripts. Northern blot analyses of extracts from protoplasts inoculated with capped *in vitro* transcripts from pSP9/55 and pSP6 showed the accumulation of negative- and positive-sense LIYV RNAs 1 and 2 and putative sgRNAs (Fig. 2B, lanes 2 and 4). In addition, there were no obvious differences in the intensity and pattern of the hybridization signals produced in extracts from protoplasts inoculated with the LIYV cDNA-derived transcripts (Fig. 2B, lanes 2 and 4) or purified LIYV virion RNAs (Fig. 2A, lanes 2 and 4). Thus, capped *in vitro* transcripts from pSP9/55 and pSP6 were infectious when inoculated together into *N. benthamiana* protoplasts and LIYV-specific RNAs accumulated to similar levels when LIYV virion RNAs were used as inoculum. We were not able to detect accumulation of negative- and positive-sense LIYV RNAs 1 or 2 or putative sgRNAs when uncapped transcripts were used as inocula (data not shown). Therefore, all further experiments described were performed with capped *in vitro* transcripts.

Although LIYV RNA 1 encodes the putative principal replicative proteins (ORFs 1a and 1b; Fig. 1A), it is conceivable that additional replication-associated proteins may be encoded within RNA 2. The closterovirus-encoded HSP70 homologue has been proposed to play a possible role in the assembly of multisubunit complexes for genome replication and/or sgRNA synthesis (Dolja *et al.,* 1994). However, our initial analysis of LIYV replication in *N. tabacum* protoplasts suggested that LIYV RNA 1 could replicate independently of RNA 2, at least in protoplasts. Even so, we could not rule out the possibility that LIYV RNA 2 was replicating at very low levels since LIYV virions and virions RNAs were used as inoculum in these studies. To attempt to determine if LIYV RNA 1 could replicate independently of RNA 2, we inoculated protoplasts with *in vitro* transcripts from pSP9/55 or pSP6 separately. Northern blot analyses of extracts from proto-

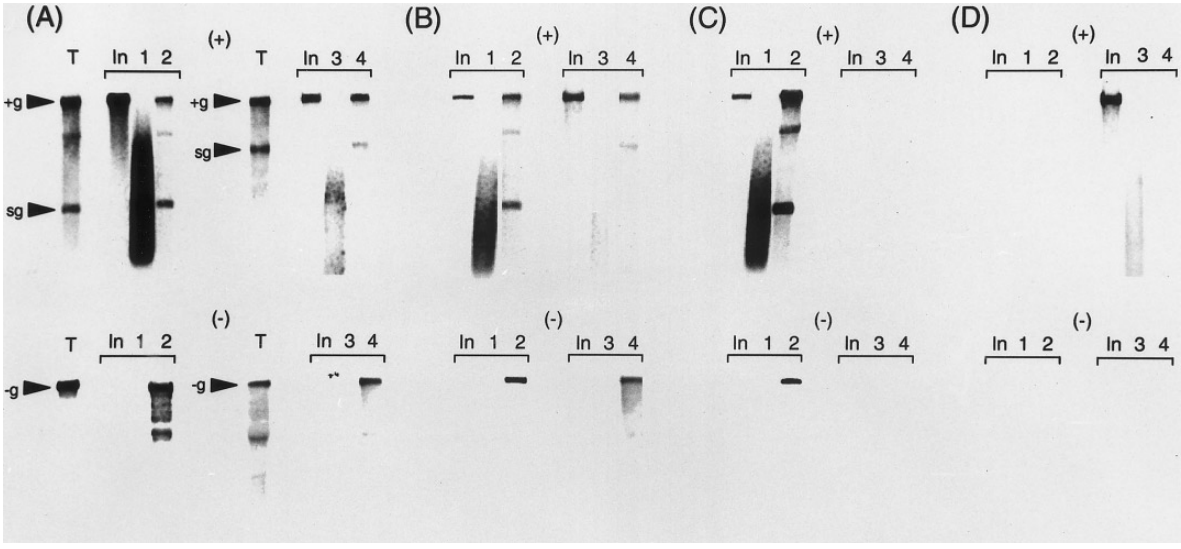

FIG. 2. Northern blot hybridization analysis of denatured total RNAs extracted from *Nicotiana benthamiana* protoplasts inoculated with (A) purified lettuce infectious yellows closterovirus (LIYV) virion RNAs 1 and 2 and capped *in vitro* transcripts of: (B) pSP9/55 and pSP6, (C) pSP9/55, and (D) pSP6. Northern blots were hybridized with DIG-labeled RNA probes to detect positive (+)- or negative (−)-sense LIYV RNA 1 (lanes 1 and 2) or 2 (lanes 3 and 4). Total RNAs were extracted from protoplasts 0.5 hr (lanes 1 and 3) and 48 hr (lanes 2 and 4) postinoculation. Each lane includes RNA from ca. 50,000 protoplasts. Inoculum RNAs (50 ng; In) and total RNAs from LIYV-infected plants (2 μg; T) are included as standards. The positions of positive (+g)- and negative (−g)-sense LIYV genomic RNAs and putative subgenomic RNAs (sg; see Klaassen *et al.*, 1995) are indicated in (A).

plasts inoculated with pSP9/55-derived *in vitro* transcripts showed the accumulation of negative- and positive-sense LIYV RNA 1 and the putative LIYV RNA 1 sgRNA (Fig. 2C, lane 2). In addition, the intensity and pattern of the hybridization signals detected in extracts of protoplasts inoculated with only pSP9/55-derived *in vitro* transcripts were similar to those detected in protoplasts inoculated with purified LIYV virion RNAs (Fig. 2A, lane 2), or with pSP9/55- and 6-derived *in vitro* transcripts (Fig. 2B, lane 2). Thus, LIYV RNA 1 can replicate in protoplasts in the absence of LIYV RNA 2, and the level to which LIYV RNA 1 accumulates at 48 hr postinoculation is not obviously different whether LIYV RNA 2 is present or absent. We were not able to detect accumulation of positive- or negative-sense LIYV RNA 2 in protoplasts inoculated with only pSP6-derived *in vitro* transcripts (Fig. 2D, lane 4). Taken together, these data suggest that the replication of LIYV RNA 2 occurs primarily via LIYV RNA 1-encoded proteins.

Accumulation of LIYV coat protein and virions in protoplasts

After determining that LIYV cDNA-derived *in vitro* transcripts were competent for replication in *N. benthamiana* protoplasts, we examined protoplasts for LIYV-encoded coat protein and virus particles. Immunoblot analysis of protoplasts inoculated with pSP9/55 and pSP6-derived *in vitro* transcripts demonstrated that LIYV coat protein was detectable at 48 hr postinoculation (Fig. 3A; lane 2). No LIYV coat protein was detected in extracts from mock-inoculated protoplasts (Fig. 3A, lane 1) or in protoplasts inoculated with only pSP9/55-derived *in vitro* transcripts (Fig. 3A, lane 3). In addition, no LIYV coat protein was

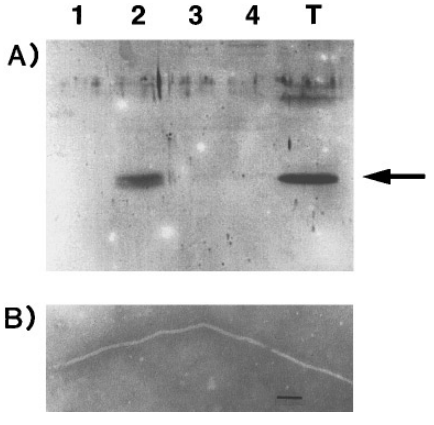

FIG. 3. Detection of lettuce infectious yellows closterovirus (LIYV)-encoded coat protein and LIYV virions in *Nicotiana benthamiana* protoplasts. (A) Immunoblot analysis of soluble proteins extracted from *N. benthamiana* protoplasts which had been mock-inoculated (lane 1) or inoculated with capped *in vitro* transcripts of: pSP9/55 and pSP6 (lane 2), pSP9/55 (lane 3), or pSP6 (lane 4). Each lane includes protein from ca. 40,000 protoplasts. Total proteins extracted from LIYV-infected *N. benthamiana* plants (T) are included as a standard. The position of the LIYV coat protein is indicated by the arrow at the right. (B) A virus particle from crude extracts of protoplasts inoculated with capped *in vitro* transcripts of pSP9/55 and pSP6. Protoplast extracts were stained with 2% phosphotungstic acid and visualized by transmission electron microscopy. The size marker (bar) indicates 82 nm.

detected in protoplasts inoculated with only pSP6-derived *in vitro* transcripts (Fig. 3A, lane 4), even though this transcript possesses the coat protein coding sequence (Fig. 1B, ORF 5). Thus, LIYV coat protein was only produced in protoplasts where LIYV RNA 2 was replicated. When crude extracts of protoplasts inoculated with pSP9/55 and pSP6-derived *in vitro* transcripts were viewed by transmission electron microscopy, typical LIYV virus particles were detected (Fig. 3B). These particles, scattered and never in groups, were indistinguishable from particles observed in protoplasts inoculated with purified LIYV virion RNAs (data not shown). No virus particles were observed in mock-inoculated protoplasts or in protoplasts inoculated with only pSP9/55-derived *in vitro* transcripts. These observations indicate that complete virion assembly occurred after inoculation of *N. benthamiana* protoplasts with pSP9/55 and pSP6-derived *in vitro* transcripts.

DISCUSSION

To date, infectious *in vitro* transcripts have been synthesized from cloned cDNAs for a wide range of unrelated positive-sense ssRNA viruses. However, the infectious *in vitro* transcripts of LIYV RNAs 1 and 2 are the first representing a closterovirus genome. This is noteworthy for several reasons. First, closterovirus genomes are large (15,311 to 19,296 nt). Although the potential to make cDNA copies of large RNA genomes has increased due to the development of DNA-synthesizing enzymes with greater polymerizing capabilities and higher fidelity, obtaining infectious *in vitro* transcripts from these large cloned cDNAs is still potentially problematic. Point mutations introduced by low-fidelity RNA polymerases during *in vitro* transcription (Kuhn *et al.,* 1990) might render these transcripts noninfectious. Infectious transcripts have been generated from cloned cDNAs corresponding to the genomes of the plant-infecting potyviruses and the animal-infecting alphaviruses (Boyer and Haenni, 1994) but these genomes are still ca. 40 and 25%, respectively, smaller than the LIYV genome.

Second, the presence of a gene encoding a HSP70 homologue is a hallmark of the closterovirus genome. Until now, the proposed functions of the viral-encoded HSP70 homologue have been primarily based on the properties of cellular HSP70 proteins. While *in vitro* studies support transport activity for the BYV-encoded HSP70, other roles for the closterovirus-encoded HPS70 can be imagined when one considers the different types of protein–protein interactions mediated by cellular HSP70s (Rothman, 1988; Craig, 1993) and the potential complexities involved in the *in planta* replication and movement of the large closterovirus genomes. Our replication assays using infectious full-length transcripts of LIYV RNAs 1 and 2 suggest that the LIYV-encoded HSP70 homologue most likely does not have a major role in LIYV RNA 1 replication in *N. benthamiana* protoplasts. However, we cannot eliminate the possibility that the HSP70 homologue may be required for *in trans* replication of LIYV RNA 2. For the bipartite cowpea mosaic comovirus (CPMV) RNAs, the *in trans* replication of CPMV M-RNA (by B-RNA encoded proteins) is dependent on the translation of the M-RNA encoded 58K protein (Holness *et al.,* 1989; Van Bokhoven *et al.,* 1993).

We have not yet tested the LIYV cDNA-derived transcripts in whole plants. LIYV is phloem-limited within its plant host (Hoefert *et al.,* 1988) and is only transmitted to plants in a semipersistent manner by the sweet potato whitefly, *Bemisia tabaci* (Duffus *et al.,* 1986). However, LIYV does replicate efficiently and assembles into virions in mesophyll protoplasts, a property also exhibited by the phloem-limited, obligately vector-borne luteoviruses. Sanger *et al.* (1994) demonstrated that beet western yellows luteovirus (BWYV) virions can be aquired *in vitro* by aphid vectors from BWYV-infected protoplasts and subsequently transmitted to plants. Our initial attempts to use this approach with *B. tabaci* and protoplasts inoculated with LIYV virion RNAs and *in vitro* transcripts were not successful and indeed, this approach may not be possible with LIYV. In contrast to the circulative-nonpropagative-transmitted luteoviruses, other semipersistent vector-borne viruses cannot be acquired as purified virions and vector-transmitted back to plants (Hunt *et al.,* 1988). However, agroinfection (Leiser *et al.,* 1992) is a probable alternative to whitefly transmission for introducing infectious transcripts into plants.

Closteroviruses such as LIYV are unique among the plant viruses in both the quantity and quality of genetic material composing their genomes, suggesting that they utilize novel means to complete their phloem-limited life cycle. In turn, how closteroviruses use their extensive genetic information to cause plant disease are important questions which must be answered in order to develop more effective means for controlling the widespread and economically important diseases they cause. The availability of infectious *in vitro* transcripts representing the LIYV genome will enable us to begin to answer these questions.

ACKNOWLEDGMENTS

We thank Elizabeth Estabrook, Tongyan Tian, Mindy Brooks, and Boni Passmore for their helpful discussions, Teresa Erickson for help with *N. benthamiana* protoplasts, and W. O. Dawson for his suggestions on this manuscript. This research was supported in part by grants from The Asgrow Seed Co. and the California Iceberg Lettuce Research Advisory Board.

REFERENCES

Agranovsky, A. A., Boyko, V. P., Karasev, A. V., Lunina, N. A., Koonin, E. V., and Dolja, V. V. (1991a). Nucleotide sequence of the 3′-terminal half of beet yellows closterovirus RNA genome: Unique arrangement of eight virus genes. *J. Gen. Virol.* 72, 15–23.

Agranovsky, A. A., Boyko, V. P., Karasev, A. V., Koonin, E. V., and Dolja, V. V. (1991b). Putative 65 kDa protein of beet yellows closterovirus is a homologue of HSP70 heat shock proteins. *J. Mol. Biol.* 217, 603–610.

Agranovsky, A. A., Koonin, E. V., Boyko, V. P., Maiss, E., Frotschl, R., Lunini, N. A., and Atabekov, J. G. (1994). Beet yellows closterovirus: Complete genome structure and identification of a leader papain-like thiol protease. *Virology* 198, 311–324.

Agranovsky, A. A., Lesemann, D. E., Maiss, E., Hull, R., and Atabekov, J. G. (1995). "Rattlesnake" structure of a filamentous plant RNA virus built of two capsid proteins. *Proc. Natl. Acad. Sci. USA.* 92, 2470–2473.

Bailey, J. W., Klement, J. F., and McAlester, W. T. (1983). Relationship between promoter structure and template specificities exhibited by the bacteriophage T3 and T7 RNA polymerases. *Proc. Natl. Acad. Sci. USA* 80, 2814–2818.

Boyer, J. C., and Haenni, A. L. (1994). Infectious transcripts and cDNA clones of RNA viruses. *Virology* 198, 415–426.

Boyko, V. P., Karasev, A. V., Agranovsky, A. A., Koonin, E. V., and Dolja, V. V. (1992). Coat protein gene duplication in a filamentous RNA virus of plants. *Proc. Natl. Acad. Sci. USA* 89, 9156–9160.

Burnett, W. N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. *Anal. Biochem.* 112, 195–203.

Candresse, T., and Martelli, G. P. (1995). Genus *Closterovirus. In* "Virus Taxonomy, Report of the International Committee on Taxonomy of Viruses" (F. A. Murphy *et al.,* Eds.), pp. 461–463. Springer-Verlag, NY.

Coffin, R. S., and Coutts, R. H. A. (1993). The closteroviruses, capilloviruses and other similar viruses: A short review. *J. Gen. Virol.* 74, 1475–1483.

Craig, E. A. (1993). Chaperones: Helpers along the pathways to protein folding. *Science* 260, 1902–1903.

Dolja, V. V., Karasev, A. V., and Koonin, E. V. (1994). Molecular biology and evolution of closteroviruses: Sophisticated build-up of large RNA genomes. *Annu. Rev. Phytopathol.* 32, 261–285.

Duffus, J. E., Larsen, R. C., and Liu, H. Y. (1986). Lettuce infectious yellows virus-A new type of whitefly-transmitted virus. *Phytopathology* 76, 97–100.

Falk, B. W., and Duffus, J. E. (1988). Ecology and Control. *In* "The Plant Viruses" (R. G. Milne, Ed.), Vol. 4, pp. 275–296. Plenum Press, New York.

Green, L. A. D., and Liem, R. K. H. (1989). *B*-Internexin is a microtubule-associated protein identical to the 70-kDa heat-shock cognate protein and the clathrin uncoating ATPase. *J. Biol. Chem.* 264, 15210–15215.

Hoefert, L. L., Pinto, R. L., and Fail, G. L. (1988). Ultrastructural effects of lettuce infectious yellows virus in *Lactuca sativa* L. *J. Ultrastruct. Mol. Struct. Res.* 98, 243–253.

Holness, C. L., Lomonossoff, G. P., Evans, D., and Maule, A. J. (1989). Identification of the initiation codons for translation of cowpea mosaic virus middle component RNA using site-directed mutagenesis of an infectious cDNA clone. *Virology* 172, 311–320.

Hunt, R. E., Nault, L. R., and Gingery, R. E. (1988). Evidence for infectivity of maize chlorotic dwarf virus and for a helper component in its leafhopper transmission. *Phytopathology* 78, 499–504.

Jones, R. W., Jackson, A. O., and Morris, T. J. (1990). Defective-interfering RNAs and elevated temperatures inhibit replication of tomato bushy stunt virus inoculated protoplasts. *Virology* 176, 539–545.

Karasev, A. V., Kashina, A. S., Gelfand, V. I., and Dolja, V. V. (1992). HSP70-related 65-kDa protein of beet yellows closterovirus is a microtubule-binding protein. *FEBS Lett.* 304, 12–14.

Karasev, A. V., Boyko, V. P., Gowda, S., Nikolaeva, O. V., Hilf, M. E., Koonin, E. V., Niblett, C. L., Cline, K., Gumpf, D. J., Lee, R. F., Garnsey, S. M., Lewandowski, D. J., and Dawson, W. O. (1995). Complete sequence of the citrus tristeza virus RNA genome. *Virology* 208, 511–520.

Klaassen, V. A., Boeshore, M. L., Dolja, V. V., and Falk, B. W. (1994). Partial characterization of the lettuce infectious yellows virus genomic RNAs, identification of the coat protein gene, and comparison of its amino acid sequence with those of other filamentous RNA plant viruses. *J. Gen. Virol.* 75, 1525–1533.

Klaassen, V. A., Boeshore, M. L., Koonin, E. V., Tian, T., and Falk, B. W. (1995). Genome structure and phylogenetic analysis of lettuce infectious yellows virus, a whitefly-transmitted, bipartite closterovirus. *Virology* 208, 99–110.

Klaassen, V. A., and Falk, B. W. (1995). Differential accumulation of lettuce infectious yellows closterovirus RNAs 1 and 2 in *Nicotiana tabacum* protoplasts. *In* "American Society for Virology," 14th Annual Meeting, July 8–12, 1995, pp. 118. [Abstract]

Koonin, E. V., and Dolja, V. V. (1993). Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences. *Crit. Rev. Biochem. Mol. Biol.* 28, 375–430.

Kuhn, R., Hong, Z., and Strauss, J. H. (1990). Mutagenesis of the 3′ nontranslated region of Sindbis virus RNA. *J. Virol.* 64, 1465–1476.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227, 680–685.

Leiser, R. M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, and Jonard, G. (1992). Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. *Proc. Natl. Acad. Sci. USA* 89, 9136–9140.

Lesemann, D-E. (1988). Cytopathology. *In* "The Plant Viruses" (R. G. Milne, Ed.), Vol. 4, pp. 220–227. Plenum Press, New York.

Murant, A. F., Raccah, B., and Pirone, T. P. (1988). Transmission by vectors. *In* "The Plant Viruses" (R. G. Milne, Ed.), Vol. 4, pp. 237–274. Plenum Press, New York.

Passmore, B. K., Sanger, M., Chin, L. S., Falk, B. W., and Bruening, G. (1993). A subviral, independently-replicating RNA stimulates the replication of beet western yellows luteovirus. *Proc. Natl. Acad. Sci. USA* 90, 10168–10172.

Rothman, J. E. (1988). Polypeptide chain binding proteins: Catalysts of protein folding and related processes in cells. *Cell* 59, 591–601.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erich, H. A. (1988). Primer-directed enzyme amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487–491.

Sanger, M., Passmore, B., Falk, B., Bruening, G., Ding, B., and Lucas, W. J. (1994). Symptom severity of beet western yellows virus strain ST9 is conferred by the ST9-associated RNA and is not associated with virus release from the phloem. *Virology* 200, 48–55.

Scholthof, H. B., Morris, T. J., and Jackson, A. O. (1993). The capsid protein gene of tomato bushy stunt virus is dispensable for systemic movement and can be replaced for localized expression of foreign genes. *Molec. Plant-Microbe Interact.* 6, 309–322.

Tollin, P., and Wilson, H. R. (1988). Particle structure. *In* "The Plant Viruses" (R. G. Milne, Ed.), Vol. 4, pp. 51–84. Plenum Press, New York.

Van Bokhoven, H., Le Gall, O., Kasteel, D., Verver, J., Wellink, J., and Van Kammen, A. B. (1993). *Cis*- and *trans*-acting elements in cowpea mosaic virus RNA replication. *Virology* 195, 377–386.